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Poly(D,L-Lactide) Nanocapsules Prepared by a Solvent Displacement Process: Influence of the Composition on Physicochemical and Structural Properties

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ABSTRACT: Nanocapsules (NC) were prepared by interfacial deposition of preformed biodegradable polymer (PLA_{50}) after a solvent displacement process. The influence of the composition used for the preparation of NC was evaluated in terms of particle size, polydispersity, zeta potential, homogeneity, and structural characteristics of the systems. The nature of the oil phase, polymer molecular weight, type and concentration of different surfactants were investigated to optimize the formulation to obtain NC suitable for intravenous administration. The influence of the physicochemical properties of the different oils used in NC preparation on the NC size was evaluated. The interfacial tension between the oil and water phases seems to have a greater effect on NC size than the oil viscosity. Miglyol 810 and ethyl oleate lead to the formation of smaller NC, probably because of the reduced interfacial tension. The polymer molecular weight plays only a small role in NC surface charge in the presence of lecithin, whereas NC surface charge, size, polydispersity, and short-term stability were highly influenced by lecithin purity. It appears that the absence of poloxamer 188 leads to smaller polydispersity, less contamination with nanospheres, and reduced formation of structures other than NC. Furthermore, electron microscopy and density gradient density techniques were used to examine the structure of the particles formed and their homogeneity. NC formation was evidenced by the bands with intermediate density between nanoemulsion and nanospheres; however, other bands of low intensity were observed. The presence of liposomes and multilayers in NC preparation was confirmed by electron microscopy. The percentage of carboxyfluorescein entrapped in different NC formulations allowed us to estimate the contamination by liposomes. It has been show that, under our experimental conditions, an excess of lecithin is an essential prerequisite for a stable preparation of PLA NC. J Pharm Sci 89: 614–626, 2000

Keywords: nanocapsules; zeta potential; drug carriers; poly(D,L-lactide); solvent displacement; electron microscopy

INTRODUCTION

The method of solvent displacement allows the production of a large variety of carriers such as nanospheres, nanocapsules, liposomes, and nanoemulsions.[1–4] Reservoir drug delivery systems like nanocapsules (NC) can be easily obtained by interfacial deposition of preformed polymers around an oily core in which lipid-soluble drugs can be incorporated. This system can reduce the inherent limitations of slow and incomplete dissolution of these poorly water-soluble drugs and

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facilitate the formation of solubilized phases from which release to the blood may occur. The association of drugs within NC allows their pharmacokinetics and distribution to be changed, which could increase their bioavailability and activity and/or reduce their toxicity.[5–8] The NC as delivery system has many advantages when a pertinent choice of drug candidates is made. The entrapment of highly lipophilic oil-soluble drugs with or without partition coefficient higher than 10^9 could reduce the release rate of these drugs and create a true reservoir drug delivery system even after intravenous injection.[9] Furthermore, the low toxicity of well-defined preformed polymers like poly (D,L-lactide) (PLA) is also interesting for the formulation of NC for parenteral routes.

Three processes for obtaining NC have been described to date: the interfacial polymerization process,[10] interfacial deposition of preformed polymers after solvent displacement,[1] and, recently, the emulsification-diffusion technique.[11] The solvent-displacement process is the simplest method that has many advantages as far as particles suitable for intravenous administration are concerned. In fact, monodispersed nanoparticles can be prepared easily in one step by simple dispersion of the nontoxic organic phase in the aqueous phase without further purification and with a very high yield of encapsulation for lipophilic substances.

NC prepared by the solvent displacement process have been studied over the last 10 years; however, not much detailed work describing their characteristics and formulation parameters used to optimize these preparations has been carried out. Some authors[12,13] have studied the mechanisms of NC formation by this process and factors influencing the size and stability of NC. Factorial design[14] was used to determine the parameters influencing the mean size of nanospheres, and they may also be applied to NC. This study indicated that the migration speed of the acetone into aqueous phase was the most significant parameter affecting mean particle size. Other important parameters studied were the concentration of the polymers in the organic phase, the ratio between the phases, and their nature. However, few data were presented concerning the influence of PLA molecular weight, nature of the oil phase (interfacial tension and viscosity), type and concentration of surfactants on the size, polydispersity, surface charge, heterogeneity, and structure of NC system formed by solvent displacement process.

In this study, the main goal was to correlate the changes in NC composition with the physicochemical and structural characteristics of the NC. The influence of the different components was initially studied in terms of size and zeta (ζ) potential to optimize NC preparation suitable for intravenous administration. Size and ζ potential of colloids are known to be the major physicochemical factors influencing the stability and the distribution of these particles after intravenous administration. Uptake of colloids by the reticuloendothelial system is known to increase as particle size and charge increases.[15] Stability is improved by reduced size and by high values of ζ potential caused by mutual repulsion of the particles. Structural evaluation of selected formulations has been undertaken. The knowledge of the inner structure, morphology, and heterogeneity of nanocapsule dispersions will contribute to the development of this drug carrier prepared by the solvent displacement process.

MATERIALS AND METHODS

Soy phosphatidylcholine, Epikuron 170® (~70% phosphatidylcholine [PC]) and Epikuron 200® (~95% PC) were purchased from Lucas Meyer (France) and Synperonic F68 (poloxamer 188) from ICI (France). Phospholipon 90® (~93% PC) was a gift from Rhône-Poulenc Nattermann (Germany). High-purity (99%) dimyristoylphosphatidylcholine (DMPC), sodium carboxyfluorescein, ethyl oleate (98%), soybean oil, and Triton X-100 were purchased from Sigma (France) and dodecane from Prolabo (France). Mineral oil (Primol 355) was supplied by Exxon (France). Miglyol 810N, 812, 829, and 840 were kindly provided by Hüls (Germany) and SPAN®80 from Fluka (Switzerland). Poly (D,L-lactide) PLA50 of MW 42 kDa, 91 kDa, and 251 kDa were supplied by Phusis (France) and of 9 kDa, 16 kDa, 109 kDa (Resomer R 202H, 203 and 206, respectively) from Boehringer Ingelheim (Germany). Sephadex® G50 coarse, Density marker beads® and Percoll® were obtained from Pharmacia (Sweden). The solvents were analytical grade, and all other chemicals were commercially available reagent grade. Water was purified by reverse osmosis (MillliQ, Millipore®).

Nanocapsule Preparation and Characterization

Nanocapsules were prepared by the method described by Fessi et al.[1] based on interfacial poly-

mer deposition after solvent displacement. PLA was used alone to prepare naked NC or associated with poloxamer 188, a hydrophilic surfactant, to prepare F68-coated PLA NC. About 60 mg of total polymer was solubilized in 2 mL of acetone and added to an acetone solution (8 mL) containing lipophilic surfactant and 250 μL of oil. This organic solution was poured into 20 mL of external aqueous phase under moderate agitation, with or without 0.375% of poloxamer 188. The solvents were evaporated to 10 mL under reduced pressure. This optimal ratio between oil/acetone/polymer/water was constant in during our experiments. NC were then analyzed without further treatment or purification. The mean size of the nanocapsules and size distribution of colloidal systems were determined by quasielastic laser light scattering, with a Nanosizer N4 Plus (Coulter Electronics Inc., FL and the ζ potential measurements with a Zetasizer 4 (Malvern Instr., UK) after dilution of NC suspensions 250 times in 1 mM NaCl (0.10 mS/cm).

Density Studies

Separation of particles was carried out on a colloidal silica gradient (Percoll® 54% v/v in NaCl 0.15 M, initial density: 1.074 g/cm^3) formed *in situ* during ultracentrifugation in a rotor model SW41Ti (Beckman) at 20°C and 20800 × *g* for 90 min 11.8 mL of Percoll were added to 0.2 mL of initial colloidal suspension without previous concentration. In a separate tube, Density marker beads® of different predeterminateddetermined densities were added under the same conditions as samples and used for external calibration of the bands. Millimeter paper strips were used to measure the distance from the top meniscus to the band limits. Particle densities were calculated from the curves plotting distance to the top versus the density of each band of marker beads.

Percentage of Carboxyfluorescein Associated with the NC Preparation

The proportion of liposomes contaminating emulsion preparations was determined by a modification of the method of Lundberg[16] using sodium carboxyfluorescein (CF) as a fluorescent water-soluble marker that could be entrapped in liposomes if these were formed. For the purpose of comparison, liposomes were obtained by the solvent displacement method similar to that used to prepare NC. The acetone phase of liposome preparations, containing only phospholipids, was heated at 40°C until total solubilization before mixing with an aqueous phase containing a self-quenching concentration of CF (50 mM). All the formulations were prepared and diluted under identical conditions. The Sephadex G50 column (0.7 × 20 cm) was loaded separately with the preparations and eluted with water to separate free CF and CF entrapped within the particles. Aliquots of total and chromatographed suspensions were analyzed after dilution in Triton X-100 0.5% to release CF. The fluorescence intensity was measured with a spectrofluorimeter (Jobin Yvon JY3D, (France) at λ_{ex} = 490 nm and λ_{em} = 518 nm. The amount of CF associated with each preparation was compared to with the amount of CF entrapped in liposomes prepared from DMPC>99% PC at the same concentration as the phospholipid in the NC formulations and to the NC prepared without lipophilic surfactant.

Transmission Electron Microscopy

Nanocapsules and pattern samples were negatively stained by floating the grids coated with a Formvar film (Fullam, France) in 2% (w/v) sodium phosphotungstate solution. Morphologic examination of particles was performed using a JEM 1200EX transmission electron microscope (JEOL, France) operating at 80KV.

RESULTS AND DISCUSSION

When the solvent displacement method is used to obtain NC, submicron oil droplets are formed and stabilized by a layer of polymer deposited at the interface, which provides a mechanical barrier to coalescence. The polymer may also alter the physico-chemical properties of the interface, for example, by the presence of hydrophilic groups. Mixtures of hydrophilic and lipophilic surfactants are generally used to further reduce size and increase stability. Fessi et al.[1] have explained the formation of nanospheres and NC by interfacial turbulence generated during diffusion of the water-miscible solvent in water. After the injection of the organic phase into water, a rapid interfacial spreading is observed as a result of the mutual diffusion between the solvents, which provides energy for oil droplet formation. This mechanical instability is caused by local variations in inter-

facial tension that can "drag" the oil into the aqueous phase.[13] Once solvent diffusion is complete, the polymer aggregates around the oil droplets. In the light of this proposed mechanism based on the Marangoni effect, we have studied some factors that are able to influence the size and the ζ potential of the NC.

Influence of the Oil Phase

The nature of the oil can play an important role in determining the physicochemical properties and the stability of the emulsion/NC systems. Under our experimental conditions, the ζ potential value was not significantly changed as a function of the nature of the oil core (−55 ± 6 mV), and no relationship could be established. Therefore, the oil seems not to be located at interface but rather completely encapsulated within the polymer. It has been reported that the volume of oil is one important parameter influencing size and viscosity of oil/water dispersions.[17,18,26] We observed an increase of size of NC as the volume fraction of the oil phase increased. Increasing the oil/lecithin ratio led to an increase in particle size (data not shown). Thus, fixed, optimal volumes of oil and lecithin (2.5% v/v and 0.3% pw/v, respectively) were used in this work to obtain NC with a submicron size by the solvent displacement method. Under these conditions, the critical parameter influencing the size of NC formed by this method is the migration rate of the organic phase, containing oil and acetone, into the aqueous phase.[13,14] This diffusion rate depends on the physical properties of the oil phase, such as viscosity and interfacial tension. Table 1 lists the values of these properties for the oils used in the NC preparations.

Quintanar-Guerreiro et al.[13] suggested that oil droplets of nanometric size are formed as a result of interfacial turbulence. The size of oil droplets formed during the diffusion process appears to be determined by the local changes in the interfacial tension during NC formation, as in an emulsification process. The magnitude of the tensioactive flux from the aqueous phase to the oil-water interface is determined by the interfacial tension at the oil-water interface during the emulsification process, which is given by eq. (1):

$$W = \gamma \Delta A \tag{1}$$

where W is the energy for emulsification, γ is the dynamic interfacial tension and ΔA is the change in the interfacial area on emulsification.[19] Thus, the lower the interfacial tension of oil, the smaller the NC size, as observed in Figure 1(A). From our results, there was a positive correlation (R^2 = 0.979) between ΔA and size. This phenomenon is in accordance with the observations of other authors.[14,20] It was found that the incorporation of poorly miscible liquids, dichloromethane or chloroform, or solutes into the acetone phase drastically increased the size of the nanospheres formed. Similar behavior was observed in the preparation of NC from oil with different interfacial tension presented in Figure 1(A). This effect is evident when the size of NC (around 200 nm) is

Table 1. Physicochemical Properties of Different Oils Used in Nanocapsule Preparation

Oil	Composition	Interfacial Tension$_{o/w}$ (mN/m)[a]	Viscosity (mPa s)[a]	Density (g/cm^3)[a]	Oil/acetone Miscibility[b] (2.5% v/v)	Ref.
Miglyol 810[c]	Caprylic/capric triglyceride	22.5	25–35	0.940–0.950	++++	21[c]
Miglyol 812[c]	Caprylic/capric triglyceride	23.0	25–35	0.940–0.950	++++	21[c]
Miglyol 829[c]	Caprylic/capric diglyceryl succinate	6.0	230–260	1.000–1.015	++++	21[c]
Miglyol 840[c]	Propyleneglycol dicaprylate/ dicaprate	19.5	8–14	0.910–0.920	++++	21[c]
Ethyl oleate		17.4 (25°C)	5.15	0.866–0.874	++++	21,23
Soybean oil		50.0	69.3	0.927	+++	21,22
Mineral oil		55–57	158	0.827–0.890	---	21,22,24
Dodecane		52.78	1.35 (25°C)	0.74–0.75	++-	22,24

[a] at 20°C.
[b] (++++ high; +++ soluble; ++- middle; --- none).
[c] According to the manufacturer (Hüls technical information, Germany).

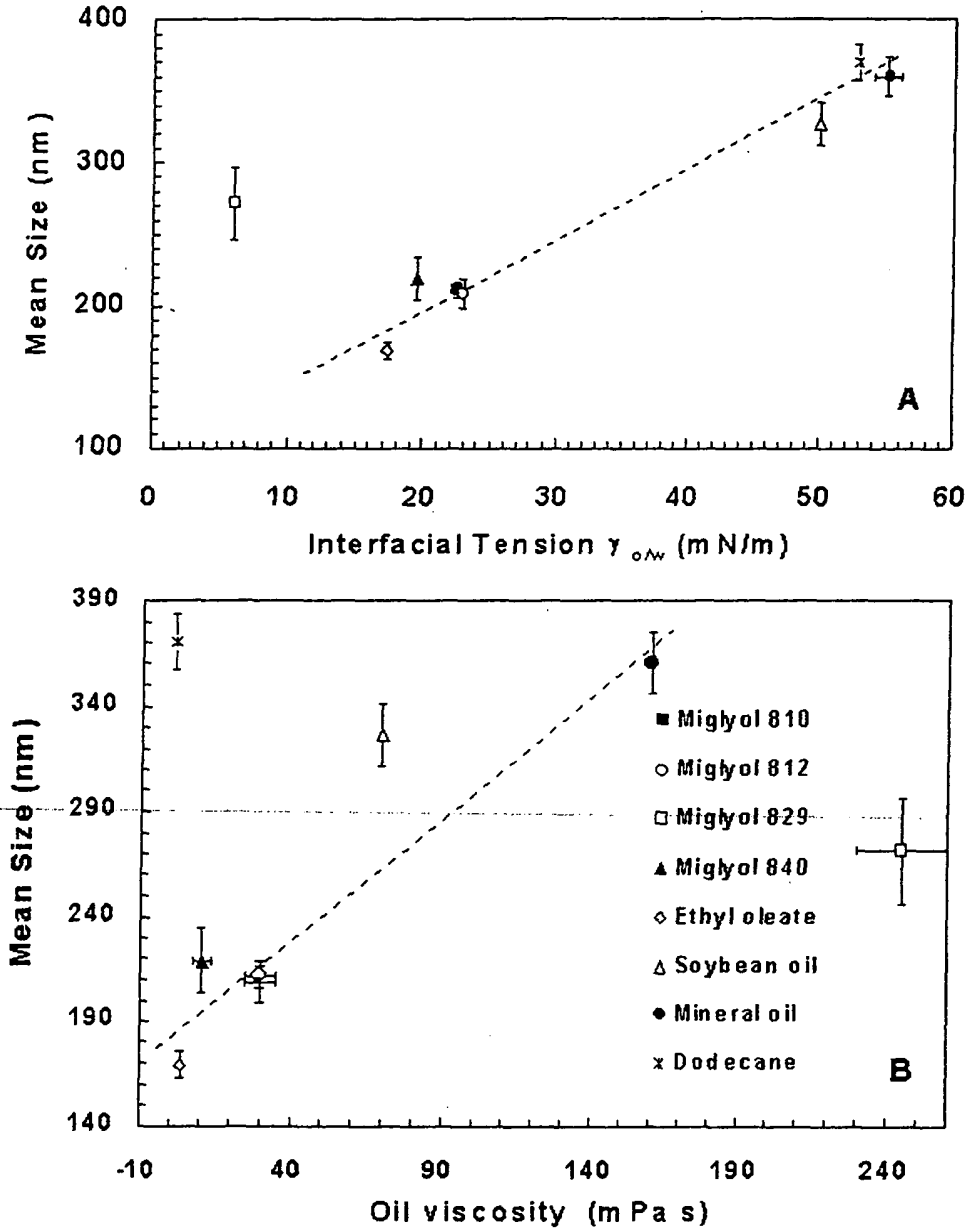


Figure 1. Influence of the interfacial tension between different oils and water (A) and the initial oil viscosity (B) on the mean size of NC. The NC were prepared with 0.3% of lecithin (Epikuron 170), 2.5% of oil phase, and 0.6 % of poly(D,L-lactide) 42,000 MW polymer at the same stirring rate. The vertical error bars represent four measurements of mean size by QELS of two NC preparations. Horizontal error bars represent the limits of interfacial tension and viscosity data taken from the literature.

compared with that of oil-free particles: nanospheres (around 100 nm) obtained by the same process. Mineral oil and dodecane have a high interfacial tension, which probably prohibits rapid spreading of the organic phase in water as a result of their reduced miscibility in acetone. Indeed, their use in our experiments increased the mean size of NC. It has been suggested that if the coalescence rate of the droplets formed is high enough to compete with the spontaneous emulsification process, an increase in the particle size may be observed.[14] This phenomenon happens when the miscibility of the solvents is reduced. Thus, it could be considered that the miscibility of an organic solvent or oil in water decreases with the increase of their resultant interfacial tension,

thus increasing the size of the nanocapsules formed.

The initial viscosity of the oil phase is another factor that could influence the mean diameter of NC. In fact, a high viscous resistance to the shear forces could increase the size of the oil droplets during the diffusion process. Figure 1(B) shows that a slightly positive correlation was observed between viscosity and particle size, but that this was less close than for interfacial tension. It should be noted that the results obtained with Miglyol 829 and dodecane were not included in the regression line shown on Figure 1(B) because they represent extreme cases: very high viscosity and low interfacial tension for Miglyol 829 and low viscosity with high interfacial tension for dodecane. The mean size of dodecane NC seems to be correlated with its interfacial tension, but not with its viscosity.

To summarize, our results suggest that the interfacial tension and viscosity of the oil are important factors that influence the particle size. No correlation could be established between oil density and NC size. Miglyol 810 and 812, both medium chain triglycerides, and ethyl oleate appear to be the most suitable oils for use in intravenous formulations because of the smaller size of the nanocapsules prepared from them. However, due to the stability of Mygliol against oxidation (≤0.5 I_2 g/100 g compared with 75 to 84 I_2 g/100 g for the unsaturated ethyl oleate), Miglyol 810 was retained for further experiments. This oil has been particularly investigated for use in total parenteral nutrition because of its safety (LD_{50} IV, 3.7 g/kg).[21]

Influence of Polymer Molecular Weight

The studies of the influence of the polymer molecular weight on the ζ potential were carried out with the two types of formulations: with or without lecithin. Measurement of ζ potential is the technique most frequently used to characterisecharacterize the surface of oil/water colloids.[25] It reflects the electrical surface potential of particles, which is influenced by the charge of the different components located at the interface with the dispersing medium. A negative charge caused by the carboxyl groups of polymer would be expected to play a role at the NC surface. In this case, the higher the polymer MW the smaller the influence on surface charge for the same weight of polymer, due to the smaller number of end groups. In the absence of lecithin, the carboxyl groups at the end of PLA chains contribute to the superficial charge, despite the presence of poloxamer, a non-ionic surfactant, as shown in Figure 2. In fact, poloxamer 188 has little influence on the ζ potential of NC; although for nanospheres it can partially mask the polymer charge (from approximately −40 mV to −1 7mV). PLA with higher molecular weights (109 and 251 KDa) yielded poorly stable NC, which were larger and susceptible to aggregation during the evaporation process. On the other hand, the results in Figure 2 indicate that the MW of the polymer has very little influence on the ζ potential of NC in presence of lecithin (Epikuron 170®). The polymer is probably masked by the charged groups of the lecithin mixture, which seems to determine the ζ potential in this case. This observation suggests that lecithin is not only located as a film on the inner surface of the polymer shell around the oil nanodroplet but also at the outer surface mixed with or surrounding the polymer film. The COOH groups from PLA polymer could contribute to NC stability for polymers of smaller molecular weights. PLA of 42 KDa MW produced NC with a mean size smaller than 200 nm and good stability and was therefore retained for further experiments.

Influence of the Surfactants

To obtain small, stable oil droplets surfactants are necessary. NC, like nanoemulsions, can be stabilized either by steric or electrostatic repulsion, depending on the nature of the surfactant. The NC prepared in this work are mixed systems that are stabilized by both poloxamer and lecithin. Figure 3 shows the influence of these surfactants on the mean diameter (A) and on the ζ potential (B) of NC.

The mean particle size of NC decreased as the Epikuron 170 concentration was increased, until a concentration of 30 mg/10 mL was reached. After this point, the diameter was not affected by further increases in the lipophilic surfactant concentration. The results indicate that to obtain stable formulations of NC with mean diameter around 180 nm, a minimal amount of soy lecithin is required, about 0.3% for the suspension containing 2.5% of oil phase. These results are similar to those obtained for the emulsions stabilized by egg phosphatidylcholine, where the same ratio oil/lecithin was found to obtain smaller sizes and

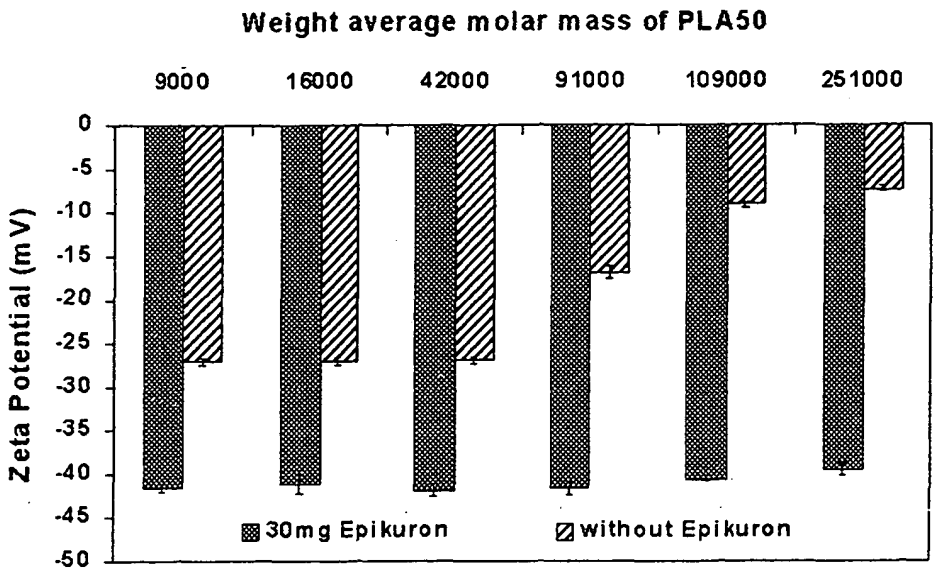


Figure 2. Effect of polymer molecular weight on ζ potential of NC. NC were obtained with 0.6% of poly(D,L-lactide) polymer of different mass molecular weights, with or without 0.3% of lecithin (Epikuron 170®), 2.5% of oil phase, and 0.3% of poloxamer 188 at the same stirring rate. Results are expressed as the mean of three measurements of two NC preparations ± S. D.

higher ζ potential values.[26] Intralipid® 10%, a fat emulsion for parenteral administration, has the same oil/lecithin ratio for droplets of 250 nm size. Stable NC were obtained without poloxamer with Epikuron concentration of 0.75%. In fact, poloxamer has little effect on reducing the size of NC at a fixed lecithin concentration. High ζ potential (greater than −30 mV) leads to stable NC suspensions because repulsion between particles prevents their aggregation. The results in Figure 3(B) show that an increase in the amount of lecithin brings about an increase in ζ potential, which reaches a plateau, probably when the oil droplets in the NC are probably completely coated by the lecithin molecules around 0.4% of lecithin. Therefore, lecithin-stabilized systems are charge-stabilized, although phosphatidylcholine, the major component, is neutral at pH 7.0. This negative surface charge (−40 to −60 mV) imparted by the lecithin is principally due to phosphatidic acid, despite its low proportion (1–2%) of the total composition of lecithins.[25] Poloxamer tends to reduce the ζ potential in the absence of lecithin until the concentration of 0.35%, above which increasing concentrations have little influence on ζ potential. As shown above, poloxamer had very little effect on ζ potential of NC in presence of lecithin.

To confirm the hypothesis that minor components of lecithin are responsible for the surface charge of NC, liposomes were prepared from lecithins of different phosphatidylcholine content. Figure 4 shows the ζ potential of these liposomes as a function of pH. The curves have the same profile, but the ζ potential value at a given pH is reduced with the purer lecithin (Epikuron 200). The higher concentration of phosphatidic acid present in Epikuron 170® is probably responsible for the lower ζ potential values.

Figure 5 shows the effect of lecithin (Epikuron 170®) on the ζ potential of different systems as a function of pH. It can be observed that the ζ potential reaches a plateau around pH 4 to 6, which corresponds to the ionization of all phosphatidyl groups; above this pH, the overall charge of the phospholipids is negative. NC, nanoemulsions, and liposomes obtained from the same lecithin present a similar ζ potential profile as a function of pH. At higher pH values NC have more negative ζ potential values than liposomes. This effect is probably due to the ionization of carboxyl groups of the polymer and free fatty acids from the oil; the latter also is present in the nanoemulsion. It is thus evident that Epikuron plays an important role in determining NC surface charge, and minor components of lecithin influence the charge of systems stabilized by them.

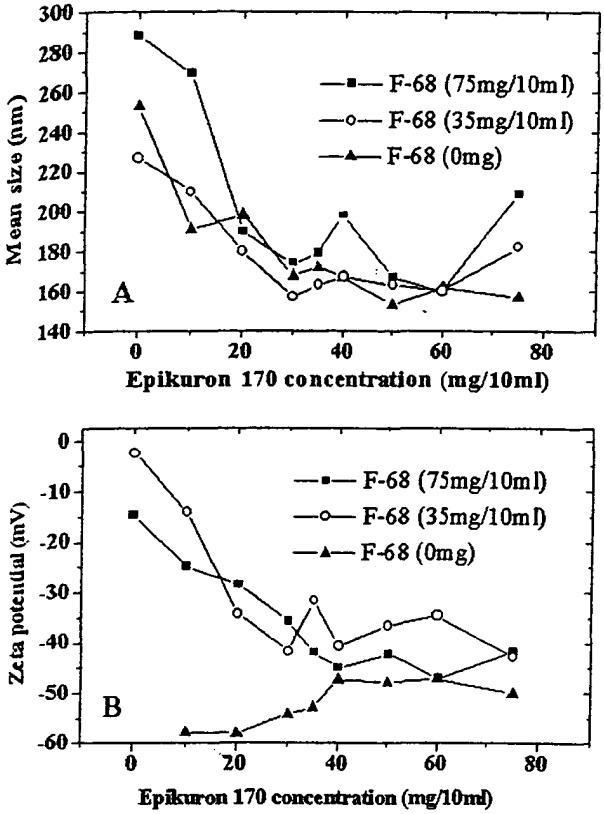


Figure 3. Effect of the increasing concentrations of lecithin (Epikuron 170®) and Poloxamer 188 (Synperonic F68®) on the mean particle size (A) and on the ζ potential (B) of nanocapsules. NC were obtained with 0.6% of poly(D,L-lactide) 42 kDa MW, and 2.5% of Miglyol 810 at the same agitation rate. Results are expressed as the mean of three measurements of two NC preparations.

Although the previous results showed that hydrophilic surfactant has only a small effect on NC short-term stability, we have observed that when large amounts of lipophilic drugs were incorporated into NC, greater amounts of poloxamer (0.75%) were needed to ensure their long-term stability.[27] The effects of further stabilization arise when hydrophilic macromolecules are adsorbed or attached on the NC surface, increasing the steric repulsion between particles and reducing interactions with biologic media.[28] Furthermore, Magalhães et al.[29] reported the enhanced adsorption of poloxamer 188 molecules in the presence of soy phospholipid monolayers at the air/water interface and suggest that it is a factor of stabilization for the systems obtained from this mixture. Poloxamer 188 molecules are capable of penetrating soy phospholipid monolayers at intermediate surface coverage with the hydrophilic

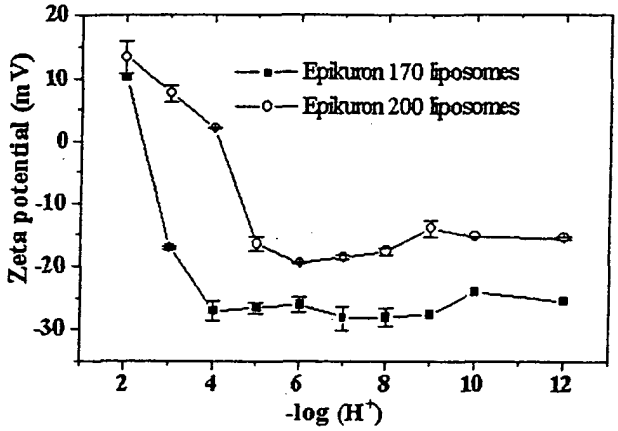


Figure 4. ζ potential of liposomes as a function of the pH, obtained with lecithin of different degrees of purity. Liposomes were obtained with 0.75% of Epikuron for both preparations at the same stirring rate. The ζ potential measurement was performed after sample dilution 50 times in solutions of different pH adjusted with NaOH and HCl at constant ionic strength. Results are expressed as the mean of three measurements of two sample dilutions (n = 6) ± S. D.

poly(ethylene oxide) moieties immersed in the water and lipid polar groups overlapping each other.[29]

Transmission Electron Microscopy Studies

The examination of TEM photomicrographs of NC indicates their tight packing (undiluted samples)

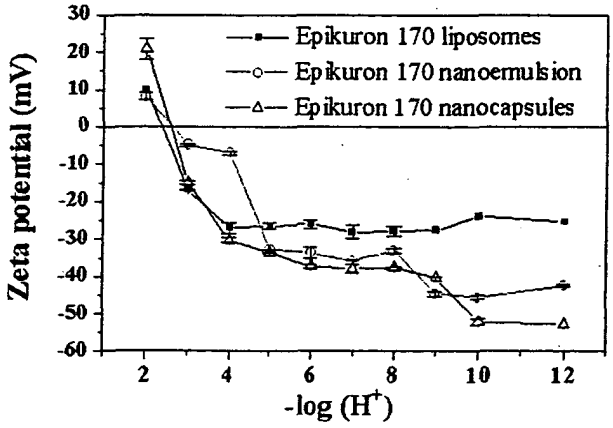
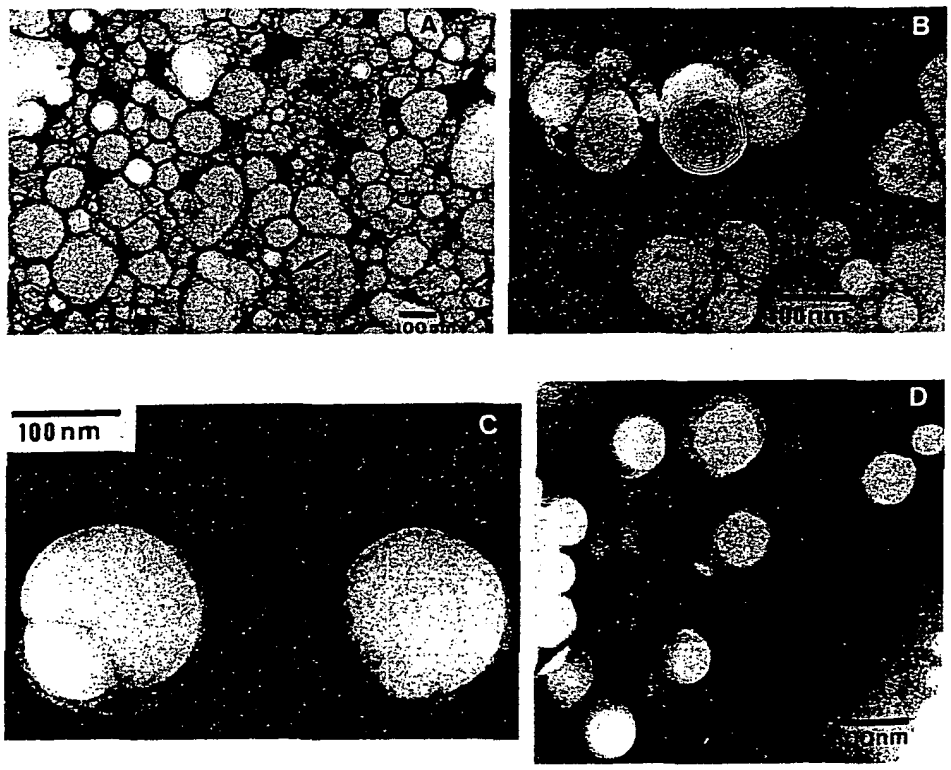


Figure 5. ζ potential of liposomes, NC, and nanoemulsion as a function of the pH, obtained with Epikuron 170® at the same concentration. Liposomes, nanoemulsions, and NC were respectively obtained with 0.75% of Epikuron, 2.5% of Miglyol 810 NC, 0.75% poloxamer 188, and 0.6% of poly(D,L-lactide) 42 kDa MW. Results are expressed as the mean of three measurements of two sample dilutions (n = 6) S. D.

and that most of the particles have structured cores [Fig. 6(A)]. A large number of particles is superimposed or hidden in this highly concentrated system. The micrographs show oil-filled structures, sometimes represented as nonstained regions [Fig. (6A)]. Two kinds of NC were analyzed: poloxamer-coated and naked NC (without hydrophilic surfactant). Poloxamer-coated NC were heterogeneous in size. Magnified micrographs show multilayered structures surrounding the particles and small liposome-like structures that co-exist with NC [Fig. 6 (A and B)], as has been reported by other authors.[30,31] These observations confirm our previous results in which the ζ potential indicated that phospholipids are present on the NC surface. A detail from a micrographs of NC prepared at higher dilution shows the low-density film surrounding the oil droplet [Fig. 6(C)]. This thin structure is probably the polymeric film. Micrographs of naked NC show a more homogeneous aspect, and no lipidic structures are detected in this case [Fig. 6(D)]. This fact could suggest that poloxamer plays a role in the formation of these complex lipid structures that contaminate NC formulations. The resulting morphology of the particles is sensitive to the sample drying and staining protocol in TEM studies. However, careful repetition of the experiments and comparison with a number of sample patterns allows us to discard the hypothesis that artifacts are responsible for these effects. TEM data lead us to propose that multilamellar liposomes are present in NC preparations containing an excess of surfactants. Some authors suggest that, for the surfactant used here, the formation of three types of structure is possible in emulsion preparations: multilamellar liposomes, oligolayers, or multilayers of surfactants at oil-water interfaces and unilamellar liposomes.[32] Multilamellar liposomes and multilayer structures were clearly identified in our NC preparations.

Density Studies

Centrifugation in a density gradient was used to confirm NC formation on the basis of the differences in density of systems formed by the solvent displacement method. The nanoemulsion (NE),



Figur 6. TEM photomicrographs of NC preparations. The undiluted NC sample (A) was obtained with 0.75% of Epikuron, 2.5% of Miglyol 810 NC, 0.75% poloxamer 188, and 0.6% of poly(D,L-lactide) 42 kDa MW; (B) detail from (A); (C) sample diluted 30 times, detail; (D) sample prepared without poloxamer and with 0.3% of Epikuron 170.

nanospheres (NS), and liposomes (LP) were prepared with the same amounts of polymer and lipids as NC. The NC density was higher than of the NE, showing the presence of the polymer at the NC interface (Fig. 7). NS have an even higher density and can be easily distinguished from NC. NS were present in the NC preparation, albeit in lower proportion. It is probable that all polymer is not deposited at the interfacial level, thus an independent precipitation could lead to NS formation. Quintanar-Guerrero et al.[11] and Chouinard et al.[33] have used the same gradient method to characterize the NC obtained by the interfacial polymerization and emulsification-diffusion techniques, respectively. They did not detect bands with densities consistent with NS in their preparation. However, both groups performed two preceding centrifugations to concentrate the samples that could have eliminated any NS present. This fact makes it impossible to conclude whether the aforementioned processes leads to formation of NS in the NC preparation. Naked NC without poloxamer lead to fainter NS bands. Theoretically, poloxamer could increase the dispersibility of the polymer in water, leading to increased formation of NS. TEM analysis confirms that naked NC without poloxamer have a more homogeneous aspect.

The NC obtained in our experiments had density values between 1.015 and 1.052 g/cm^3. These densities are intermediate between NE and NS. No separate oil phase was detected in the supernatant after ultracentrifugation of NC. Taking the analysis of TEM micrographs and NC density together, it can be concluded that the polymeric film is thin; less than 10 nm. This fact can be explained by the high surface developed in NC formulations compared with the surface developed in other systems (NS for example), leading to a smaller amount of polymer per particle. The analysis of electron micrographs and particles density confirms the formation of NC. Liposome

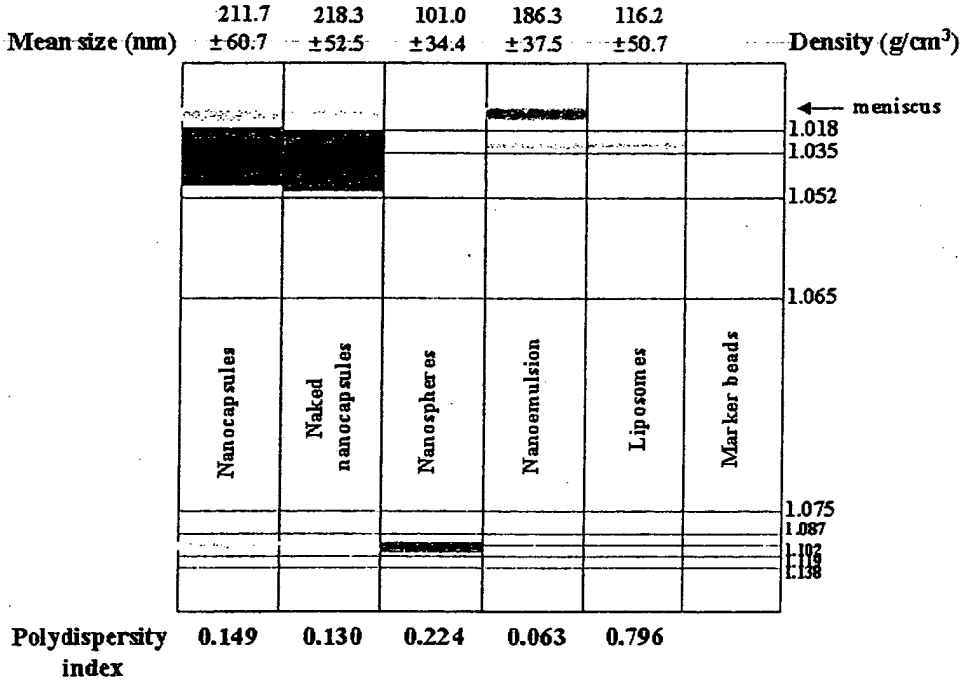


Figure 7. Ultracentrifugation of different formulations in Percoll gradient. Gradient density was monitored using colored density marker beads in 0.15 M NaCl. The starting density was 1.074 g/cm^3. The sizes given are the means and standard deviation of populations that were reported by the instrument (n = 4) from two individual preparations. NC were obtained with 0.75% of Epikuron 170, 2.5% of Miglyol 810 NC, 0.75% poloxamer 188,m and 0.6% of poly(D,L-lactide) 42 kDa MW, naked-NC without poloxamer, nanoemulsion without polymer, nanospheres without Epikuron and oil, and liposomes with 0.75% of Epikuron 170 only. The gray-scale intensity of the bands shown on the Figure is proportional to the intensity of the bands in the gradient.

density seems to be near that of NC, a fact that prevents their separation by this method. It is interesting to note that liposomes are also present in nanoemulsion and NC preparations as described by some authors.[30–32] Thus, the results of these different techniques (electron microscopy and density gradient centrifugation) are complementary and in agreement with one another.

Proportion of Liposomes in NC Formulations

To confirm the observations reported above suggesting that an excess of PC within the formulation could form vesicles or multilayers around the particle interface, a hydrophilic fluorescent marker, CF, was included in the preparation. This marker would be encapsulated within closed vesicles with an aqueous core but would not be incorporated into NC. Thus, any marker found in the void volume after size exclusion chromatography would indicate the presence of phospholipid vesicles in the preparation. Table 2 shows the percentage entrapment of CF in NC prepared with different lipophilic surfactants and compares this with the entrapment in liposomes obtained with different phospholipids alone by the solvent displacement method. A significant amount of entrapped CF was found in NC preparations when higher purity phosphatidylcholine was used as the lipophilic surfactant for the oily phase, even at a low lecithin concentrations (0.15%), indicating that a large aqueous compartment is formed, probably lipid meso and lamellar phases. On the other hand, no CF could be detected to be associated with NC formed in the absence of lecithin. When higher amounts of lecithin were used (0.75%), the percentage of CF entrapped increased, showing increasing contamination of the NC system with closed lipid structures. When PC was replaced by other surfactants, the percentage of liposome contamination as estimated by entrapped CF decreased. Only SPAN 80® produced relatively good short-term stability of NC with low contamination by liposomes, although after 1 month the preparation showed signs of instability. The NC prepared with monoacylglycerols showed macroscopic precipitation of monoglyceride components during solvent evaporation,

Table 2. Effect of Different Lipophilic w/o Surfactant on Particle Size, Stability, and Proportion of Carboxyfluorescein Entrapped in Nanocapsule of Poly(D,L-Lactic Acid) MW 42,000 Preparations

	Composition[a]	Oil/ Surfactant mg/ml	% Entrapped CF	Average Diameter nm[b]	Poly-Dispersity Index[c]	ζ Potential (mV)[d]	Stability[e]
Liposomes	DMPC >99%	7.5	7.05	121 ± 48	0.422	−26.5 ± 0.2	nd
	Epikuron 200	7.5	3.25	149 ± 54	0.282	−39.7 ± 0.2	nd
	Epikuron 170	7.5	3.40	126 ± 55	0.767	−60.3 ± 0.4	nd
	Phospholipon 90	7.5	2.24	122 ± 42	0.222	−32.2 ± 0.7	nd
Nanocapsules	Miglyol	25/0.0	<0.01	243 ± 65	0.104	−25.5 ± 0.1	+++++ (creaming)
	Miglyol/DMPC >99%	25/7.5	5.54	682 ± 313	1.328	−28.2 ± 0.3	++--- (creaming)
	Miglyol/DMPC >99%	25/1.5	1.08	888 ± 415	1.726	−29.3 ± 1.9	++--- (creaming)
	Miglyol/Epikuron 200	25/7.5	8.40	337 ± 146	0.772	−38.6 ± 0.1	+----
	Miglyol/Epikuron 200	25/1.5	1.55	217 ± 63	0.134	−35.3 ± 0.5	+++++
	Miglyol/Epikuron 170	25/7.5	1.96	176 ± 65	0.303	−53.3 ± 1.5	+++++
	Miglyol/Epikuron 170	25/1.5	0.77	190 ± 55	0.127	−33.3 ± 0.5	+++++
	Miglyol/Phospholipon 90	25/7.5	4.47	241 ± 77	0.173	−32.2 ± 0.2	++--- (Sedimentation)
	Miglyol/Phospholipon 90	25/1.5	2.12	225 ± 65	0.126	−33.5 ± 0.1	+++--
	Miglyol/SPAN 80	25/5.0	<0.01	208 ± 51	0.086	−27.9 ± 0.3	+++++ (flocculation)
	Miglyol/glyceryl monolaurate	25/2.0	1.07	219 ± 45	0.080	−19.5 ± 0.7	+---- (Sedimentation)
	Miglyol/glyceryl monostearate	25/2.0	1.51	240 ± 70	0.133	−21.3 ± 0.9	+---- (Sedimentation)

[a] 0.6% of polymer and 2.5% of Miglyol 810N were used in NC preparation.
[b] Standard deviation of populations that were reported by the instrument (n = 4).
[c] Monodispersed samples (<0.3).
[d] For ζ potential measurement the suspensions were diluted 250 times in NaCl 1 mM (100 μS/cm).
[e] Evaluated during 60 days by changing in mean size, visual creaming, flocculation, and sedimentation.

which suggest that they were not incorporated in the preparation (or at the interface). The NC without lipophilic surfactant and those obtained with monoglycerides (in the supernatant) showed similar size distribution, polydispersity, and ζ potential. Thus, monoglycerides are not effective as lipophilic surfactants for the NC system, although they are largely used as auxiliary lipophilic surfactants for stabilizing oil/water emulsion systems.

The excess of phospholipids in the NC preparation could lead to the formation of lamellar phases or multilayer arrangements, depending on the amount of water, nature, and three-dimensional molecular shape of phospholipids.[32] Normally, to accommodate the additional phospholipids, an increased interfacial area is produced, leading to smaller particle sizes in emulsions. However, when the radius of curvature of oil droplets reaches a particular low value, the phospholipids may no longer energetically favor further decreases in particle sizes. At this point the phospholipids form other structures. It is interesting to note that an increase in lecithin purity increases the size and polydispersity index of the NC in contrast to the effect obtained for liposomes prepared from phospholipids alone. Pure phospholipids are not ideal for preparing NC with a diameter lower than 200 nm. NC obtained from the most pure lecithin had increased mean size and polydispersity index and lower stability. As previously shown in this work, minor components of lecithin are essential for NC stabilization by increasing NC surface charge. The higher level of entrapped CF in some preparations of NC generally coincides with more purified emulsifier (DMPC or Epikuron 200®).

In previous studies, the determination of the minimum amount of emulsifier needed for the preparation of stable NC in the micrometer range indicated that several monolayers of emulsifier are required.[30,31] Approximate calculation of the minimal amount of lecithin (considering a monolayer of phosphatidylcholine and an oil droplet of 170 nm) necessary to completely cover the total oil droplet surface as a monolayer leads to an Epikuron170® concentration of 0.15% for the NC formulations used in this work. It therefore seems that an equilibrium exists between lecithin at the NC surface and in the form of independent vesicles (because at 0.15% vesicles are already present, and 0.30% is necessary for optimum NC formation).

CONCLUSION

A wide range of carriers can be prepared with a solvent displacement process, as has been shown in this work. In summary, we have investigated various NC formulations of varying composition. The interfacial tension between oil and water phases has a preponderant role in determining NC size. We have tried to highlight the physicochemical and structural importance of lecithin and poloxamer for NC formation and stabilization. Density and morphologic studies by TEM of optimized NC formulations are in agreement concerning NC formation and the presence of organized lipid complex phases. Finally, the studies with incorporated CF were useful to confirm the formation of lipid vesicles and to estimate the extent of their contamination in NC preparations. The results obtained in our work show that an increase in stability is accompanied by the formation of both NC and vesicles in the same preparation process. NC are multicomponent systems, and the development of an optimal formulation should combine stability with a monodisperse, homogeneous preparation. However, their potential as high payload carriers for lipophilic drugs makes them worthy of further study.

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